

**Immune Response in American Quarter Horses in Relation to Vaccination
Time**

Thesis

Partial Fulfillment of the Requirements for Undergraduate Research Distinction

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ABSTRACT

Circadian rhythms have been reported to influence immune responses in both humans and animals. Studies in mice have shown that serum antibody concentrations were higher when vaccinated in the evening and a recent study in horses suggests that humoral responses may be increased when antigen exposure occurs in the evening. These studies highlight the potential for time of day to influence immune response to vaccination. Increasing antibody response to vaccination simply by changing the time of day of vaccination may translate into increased vaccine efficacy and improved horse health. In this study, eight Quarter Horse mares (10.5 ± 5.8 yr) were used to evaluate the time of day of vaccination on serum IgA, IgM, IgG, IgGa, IgGb, and IgG(T) concentrations in response to vaccination. Mares were randomly assigned to one of two vaccination groups: AM or PM. All mares received 0.05% BW of a 12% CP pelleted concentrate with mixed grass hay and water *ad libitum* and were housed in outdoor paddocks with access to shelter at all times. Mares in the AM vaccination group were vaccinated at 0700 hr. Mares in the PM vaccination group were vaccinated at 1900 hr. All mares were vaccinated against Eastern and Western equine encephalomyelitis, equine rhinopneumonitis (EHV-1 and EHV-4), equine influenza (type A2), tetanus and West Nile virus. Blood samples were taken via jugular venipuncture at 1300 hr immediately prior to vaccination (d 0) and on d 7, 14, 21 and 28 post-vaccination. Sera samples were measured for IgG, IgGa, IgGb, IgG(T), IgA and IgM specific antibodies using commercial ELISA kits. Data were analyzed using the PROC MIXED procedure of SAS with d 0 as a covariate and a P value of ≤ 0.05 was considered statistically significant. The immunoglobulins evaluated in this study differed in their response to time of day of vaccination. IgA concentrations increased in response to vaccination and tended to be higher in the PM vaccination group ($P = 0.07$). There were no differences in IgM, IgGa or

IgG(T) concentrations between the mares in the AM and PM vaccination groups. Mares in the PM vaccination group had higher IgGb concentrations on d 7, 14, 21 and 28 d post-vaccination ($P < 0.01$). However, total IgG concentrations were only increased in the PM vaccination group on d 21 post-vaccination ($P < 0.01$). Although time of day of vaccination influenced some of the immunoglobulins evaluated in this study, there are additional immune responses that need to be investigated. Further research is needed in this area to determine the optimum time for vaccination.

Keywords

Time of Day, Vaccination, Immunoglobulins

INTRODUCTION

Circadian rhythms have been reported to influence immune responses in both humans and animals (Petrovsky et al., 1997; Cernysiov et al., 2009). The circadian system provides animals with a means to adapt internal physiology to constantly changing environmental stimuli (Murphy, 2007). The immune system is essential in protection against infectious agents to which the horse may be naturally exposed and immunoglobulin production in response to antigens is essential for horse health. The interaction of immunoglobulins in response to vaccination at different times of day has received limited attention in horses. Previous research has reported that immunoglobulins in peripheral blood differentially responds to antigenic challenge over a 24-h cycle and this can impact vaccination management practices in horses (McGlynn et al., 2010) as well as influencing our understanding of the immune response to vaccination. Production of immunoglobulins by the horse can be influenced by a variety of factors including environment, age, diet, stress and disease.

Immunoglobulins (Ig) are glycoproteins that are produced by plasma cells after stimulation by an antigen. They are also referred to as antibodies. In horses, there are five major Ig classes that have been described; IgG, IgM, IgA, IgD and IgE (Horohov, 2014). Within the IgG classes there are seven different subclasses, IgG1-IgG7 (Keggan et al., 2013). IgG1 and IgG2 correspond to IgGa, IgG3 corresponds to IgG(T), IgG4 corresponds to IgGb, IgG5 corresponds to IgG(T), and IgG6 and IgG7 correspond to IgGc (Wagner, 2005).

IgG is the most prevalent immunoglobulin in equine serum and colostrum (Sheoran et al., 2000) and is also found in mucosal surfaces such as the urinary tract, lower respiratory tract and lung (Butler, 1998). In colostrum, IgGb is the most prevalent immunoglobulin followed by IgGa and IgG(T). IgGa and IgGb are also detectable in nasal wash samples (Sheoran et al., 2000). The systematic antibody response, along with mucosal IgG antibody response is known to play a vital role in protection against equine pathogens like equine influenza virus (Nelson et al., 1998; Breathnach et al., 2006).

IgM is the major immunoglobulin produced during a primary immune response. It is also produced in a secondary response but it is usually masked by the predominance of IgG (Tizard, 2000). IgM is the first isotype expressed during B-cell development and during a primary immune response to an infection (Fermaglich, 2003). IgM is a major antibody of the mucosal immune response, and the only immunoglobulin that is more prevalent in mucosal immune response is IgA.

IgA is mostly found in the mucosa, saliva, tears and sweat of the horse. IgA can opsonize antigens that are later phagocytized by macrophages located on mucosal surfaces (Cunha et al., 2006). IgA works closely with IgE such that if IgA production is deficient, then an IgE response

may trigger excess amounts of IgE to be produced (Tizard, 2000). As a result of this balancing act, low levels of IgA result in increased IgE production and can also lead to the development of allergic responses to food or inhaled antigens (Tizard, 2000).

The goal of vaccination is to prime the humoral and cellular immune responses without causing disease. In vaccinating a horse, an antigen is used to stimulate a humoral response. An effective vaccine should prevent disease and virus shedding from the animal (Gildea et al., 2011). Studies in mice have shown that serum antibody IgM and IgG concentrations were higher when vaccinated in the evening (Cernysiov et al., 2009). A recent study in horses suggests that humoral responses may be increased when antigen exposure occurs in the evening (McGlynn et al., 2006). These studies highlight the potential for time of day to influence immune response to vaccination. Increasing antibody response to vaccination simply by changing the time of day of vaccination may translate into increased vaccine efficacy and improved horse health. The objective of this study is to determine if time of day vaccination influences immunoglobulin response in horses.

MATERIALS AND METHODS

Horses - Eight Quarter Horse mares (10.5 ± 5.8 yrs.) were randomly assigned to one of two vaccination groups: AM or PM. All horses were housed outside in grass paddocks and received 0.05% of body weight (BW) of a 12% CP pelleted concentrate, supplemental mixed grass hay, and water *ad libitum*.

Vaccination and Serum Collection – Horses were vaccinated intramuscularly with Fluvac Innovator® 5 (Eastern and Western encephalomyelitis, rhinopneumonitis (EHV-1 and EHV-4),

influenza type A2 and tetanus) and West Nile Innovator® (West Nile Virus). Horses in the AM group were vaccinated at 0700 hr and horses in the PM group were vaccinated at 1900 hr. Blood samples were collected via jugular venipuncture immediately prior to vaccination (d 0) and then on d 7, 14, 21 and 28 post-vaccination at 1300 hr. Blood samples were placed into BD Vacutainer 10 ml red top tubes and centrifuged at 2500 rpm for 10 min. Serum was decanted and stored at -80°C until further analysis.

ELISA Kits - Serum samples were evaluated by the use of commercially available kits for equine serum: Horse IgA ELISA Quantitation Set; Bethyl Laboratories Cat. No. E70-116, Horse IgM ELISA Quantitation Set; Bethyl Laboratories Cat. No. E70-114, Horse IgGa ELISA Quantitation Set; Bethyl Laboratories Cat. No. E70-124, Horse IgGb ELISA Quantitation Set; Bethyl Laboratories Cat. No. E70-127, Horse IgG(T) ELISA Quantitation Set; Bethyl Laboratories Cat. No. E70-105, and four parameter logistics curves. Serum samples were diluted in order to fit the curve set by the standards. Standards and unknown samples were assayed in duplicate for each ELISA. All duplicate values were within 5% of each other. The intra-assay coefficient of variation was less than 3.01%, the inter-assay variation less than 2.37%, and the minimal detectable concentration for IgA, IgM and IgG(T) was 15.6 ng/ml and IgGa and IgGb was 3.12 ng/ml.

Data Analysis - Data were analyzed using MIXED procedure of SAS v 9.3 (SAS Institute Inc., Cary, N.C.). The model included the fixed effects of vaccination time, day of sampling and 2-way interaction, and the random effects of horse nested within vaccination time. The repeated measures on horses were modeled using either the best structure of error. The decision in regards

to the best error structure was made based on the smallest Bayesian Information Criterion. Comparisons of the AM vaccination time and PM vaccination time across all sampling days were made by decomposing the vaccination time by day term into single degrees of freedom contrasts. Other mean comparisons were made using Fisher's protected least-significant difference (LSD). Significance was declared at $P \leq 0.05$. All results are expressed as least-square means with their respective standard errors of the least means square.

RESULTS

Serum IgGa Concentrations

Serum IgGa concentrations between AM Vaccination group and PM Vaccination group are shown in Figure 1. The mares in the AM Vaccination group showed a typical immune response after vaccination, peaking at d14 then decreasing over time. However, the PM Vaccination group had little change from d7 through d21; and at d28, IgGa concentrations decreased. The mares in both the AM and PM Vaccination groups appeared to have peak IgGa production at d14. Overall, there were no differences between AM and PM vaccination groups ($P = 0.08$).

Serum IgGb Concentrations

Serum IgGb concentration between AM and PM Vaccination group are shown in Figure 2. The mares in both the AM and PM Vaccination groups had peak IgGb levels at d14 then decreased steadily over time. The PM Vaccination group had a greater response in IgGb concentrations on d7 ($P = 0.0057$), d14 ($P = 0.0329$), d21 ($P = 0.0104$), and d28 ($P = <0.0001$). Overall, differences between AM and PM vaccination times were significant ($P = 0.0037$). Mares

in the PM vaccination group had higher IgGb concentrations on d 7, 14, 21 and 28 d post-vaccination ($P < 0.01$).

Serum IgG(T) Concentrations

Serum IgG(T) concentration between AM Vaccination group and PM Vaccination group are shown in Figure 3. Mares in the AM Vaccination group showed an increase in IgG(T) concentrations after vaccination, peaking at d14 then decreasing over time. The PM Vaccination varied in IgG(T) response with d14 having the lowest production of IgG(T) and d7 and d21 having almost the same production levels of IgG(T) in response to vaccination. Overall, differences between vaccination time were not significant ($P = 0.3214$).

Serum IgG Concentrations

Total serum IgG (IgGa,IgGb,IgG(T)) concentrations between the AM and PM Vaccination groups are shown in Figure 4. The AM Vaccination group shows an increase in total IgG concentrations after vaccination, peaking at d14 then decreasing over time. In contrast, total IgG concentrations remained relatively constant in the PM Vaccination group until 28d post-vaccination. The PM Vaccination group on d21 was significantly higher in IgG compared to the AM Vaccination group ($P = 0.0094$).

Serum IgM Concentrations

Serum IgM concentration between AM and PM Vaccination groups are shown in Figure 5. The mares in the AM Vaccination group showed little variation in IgM concentrations throughout the study. Although mares in the PM Vaccination group showed a slight increase in IgM 14d post-vaccination, there were no differences in IgM concentrations due to the time of day of vaccination.

Serum IgA Concentrations

Serum IgA concentrations between AM and PM Vaccination groups are shown in Figure 6. IgA concentrations increased in response to vaccination and tended to be higher in the PM vaccination group ($P = 0.07$). Both the AM and PM Vaccination groups had a similar pattern of having decreased IgA concentrations on d14 then increasing on d21 then decreasing again on d28.

DISCUSSION

Overall, the levels of immunoglobulins seen in the present study were consistent with previous studies conducted in mature horses. The typical range for IgG in mares was 2463.9 ± 1337.3 mg/dL, IgM was 136.4 ± 218 mg/dL and IgA was 305.2 ± 237.5 mg/dL (Kohn et al., 1989). IgGa, IgGb, and IgG(T) concentrations tended to be slightly higher than previous recorded levels. A possible reason of these values being higher than normal is due to the fact, IgG (IgGa, IgGb, IgG(T)) plays a role in neutralizing immune responses to the equine herpesvirus type 1 (EVH-1), EVH-4, and equine influenza (Keggan et. al, 2013). All horses were vaccinated against these diseases.

Lewis et al. (2008) states that for maximum vaccine efficacy, the vaccine should elicit an antibody response in IgG1 (IgGa), IgG3 (IgG(T)), IgG4 and IgG7 (both IgGb) subclasses. In the present study, IgG1, IgG3, IgG4 and IgG7 all had a typical immune response to vaccination with a peak at d14 then decreasing over d21 through d28. However, the only subclass of IgG that was influenced by time of vaccination was IgGb. Previous research indicates that IgGb is the most prevalent of all IgG subclasses in equine serum followed by IgGa (Sheoran et al., 2000). However, in the present study, IgG(T) was much more abundant than IgGa or IgGb . The

vaccine used in this study included a tetanus toxoid antigen, which could have caused the increase in IgG(T) concentrations (Weir et al., 1966; Widder et al., 1986).

IgA is predominantly a secretory immunoglobulin, found in much higher concentrations in nasal swabs than in equine serum (Sheoran et al., 2003). Therefore, different patterns may have been observed in response to vaccination if nasal swabs were taken and analyzed along with the serum samples. Similar to previous research, IgA concentrations in the current study fluctuated slightly with a decrease from d7 to d14 and an increase from d14 to 21 followed by a rapid decrease from d21 to d28 (Koke, 2014).

When evaluating immunoglobulin concentrations in response to time of day of vaccination, the PM Vaccination group tended to have greater IgGa, IgGb, IgM and IgA concentrations compared to the AM Vaccination group. A study performed by Cernysiov et al. (2009) observed that the antibody concentrations of IgG and IgM were significantly higher when the mouse was immunized in the evening. The authors later considered this increase in antibody concentrations in the evening could be linked to melatonin.

IgM concentrations were the lowest in response to vaccination throughout the study. This could be due to IgM being the first immunoglobulin expressed during B-cell development and during a primary immune response (Fermaglich, 2003). IgM is also produced in a secondary response but it is usually masked by the predominance of IgG (Tizard, 2000). Since the horses in this study had previously been vaccinated against the same antigens, their immune response would be considered a secondary response. The increase in IgM concentrations would have occurred much sooner than when sampling took place. If sampling occurred sooner than d7, we may have observed a greater increase in IgM levels. IgG(T) appeared to show an opposite trend

than what was seen in IgGa and IgGb. This may be partially due to IgG(T) levels being inversely related to IgG levels in equine serum (McGuire et al., 1971).

CONCLUSION

This study suggests that the time of day of vaccination may influence humoral immune responses in horses, with increased immunoglobulin concentrations occurring when the horse is exposed to antigens in the evening. Further research is needed in this area to determine the optimum time for vaccination.

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FIGURES

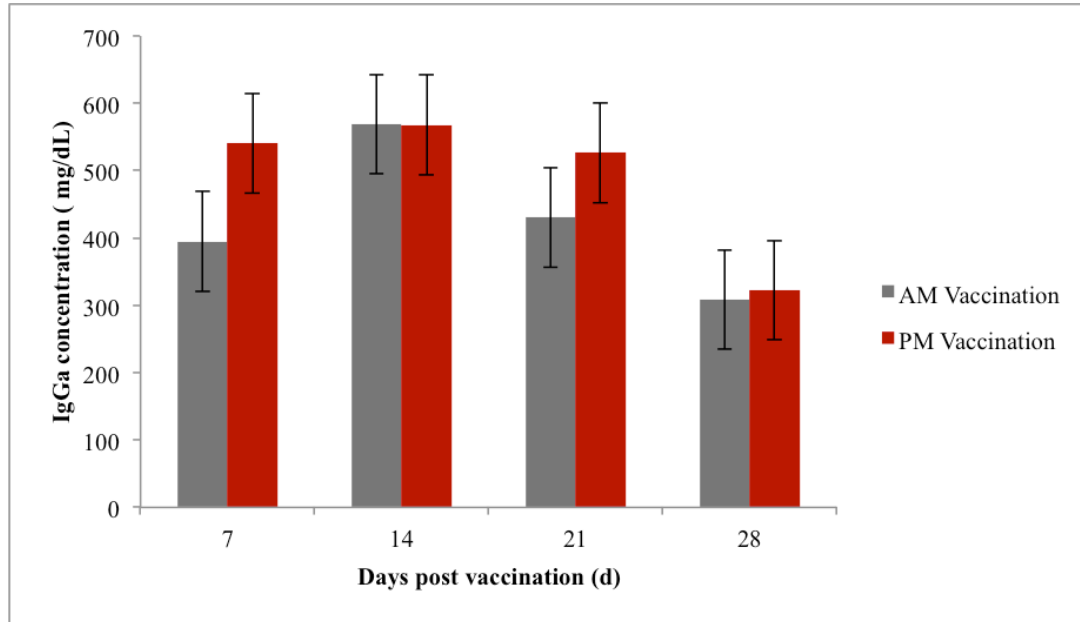


Figure 1: Serum IgGa concentrations in mg/dL from D7 through D28 post vaccination (n=4 per time point per group). Bars represent least square mean \pm SE. Day P=0.05; Vaccination Time (VxTime) P=0.08; Day*VxTime P=0.78.

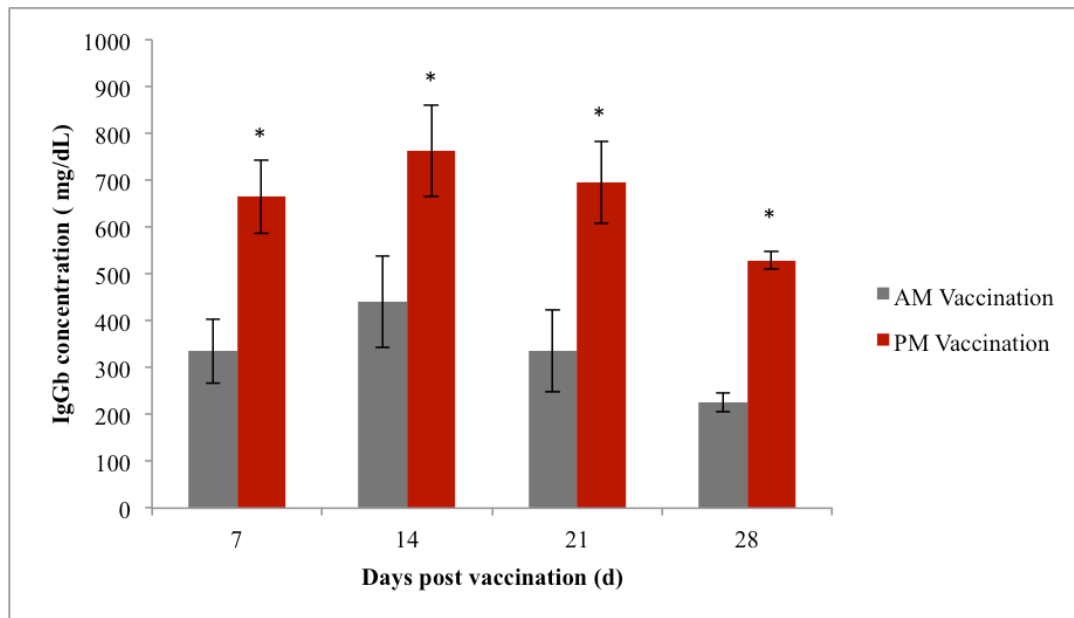


Figure 2: Serum IgGb concentrations in mg/dL from D7 through D28 post vaccination (n=4 per time point per group). Bars represent least square mean \pm SE. Day P=0.0072; Vaccination Time (VxTime) P=0.0037; Day*VxTime P=0.9711. A * denotes the value is statistically significant. *D7 P=0.0057; *D14 P=0.0329; *D21 P=0.0104; *D28 P=<0.0001.

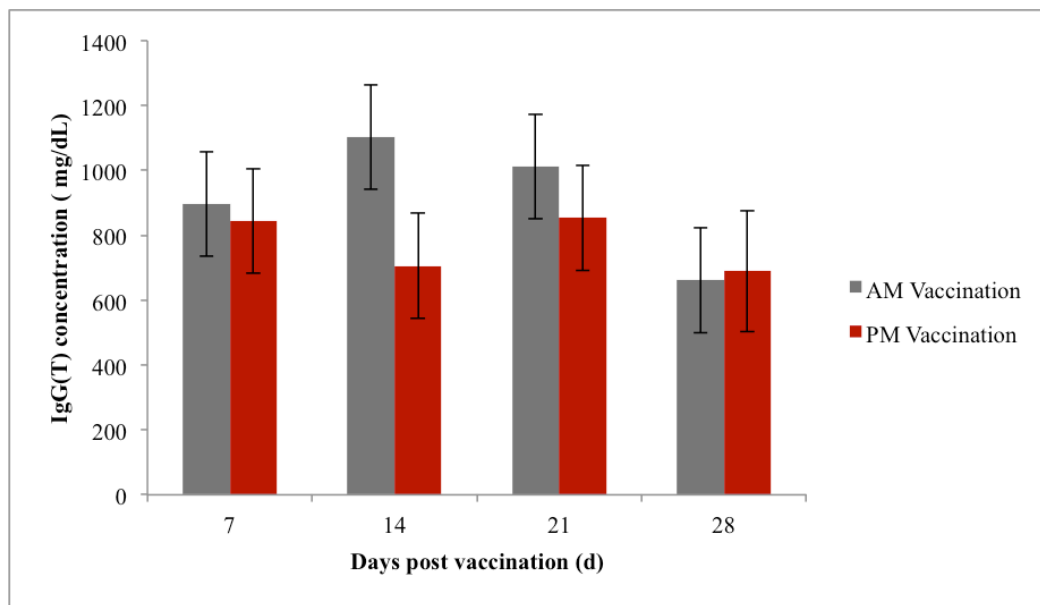


Figure 3: Serum IgG(T) concentrations in mg/dL from D7 through D28 post vaccination (n=4 per time point per group). Bars represent least square mean \pm SE. Day P=0.4128; Vaccination Time (VxTime) P=0.3214; Day*VxTime P=0.5709.

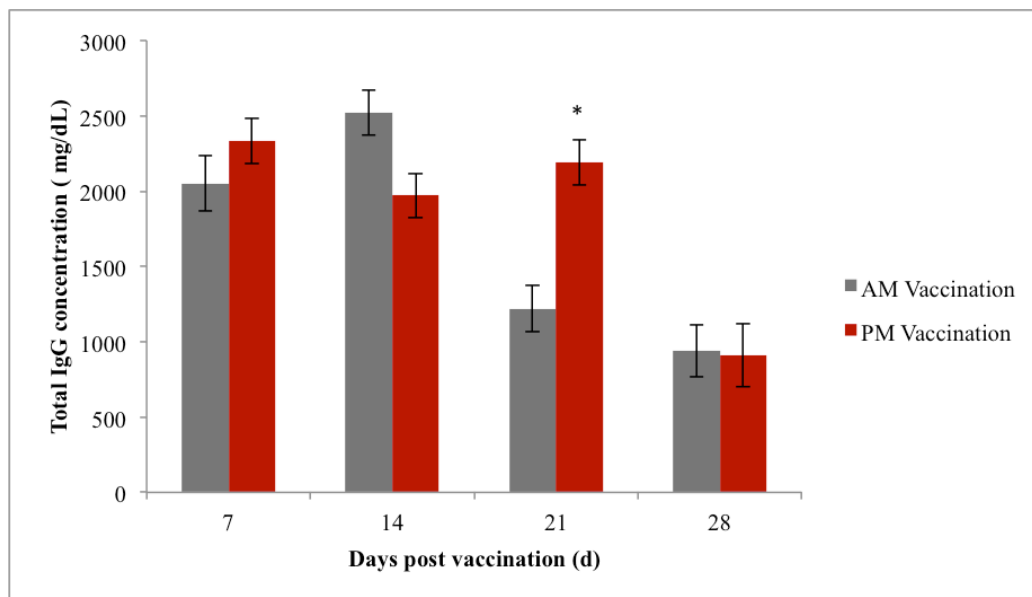


Figure 4: Total serum IgG (IgGa, IgGb, IgG(T)) concentrations in mg/dL from D7 through D28 post vaccination (n=4 per time point per group). Bars represent least square mean \pm SE. Day P=0.0061; Vaccination Time (VxTime) P=0.4141; Day*VxTime P=0.0029. A * denotes the value is statistically significant. *D21 P=0.0094.

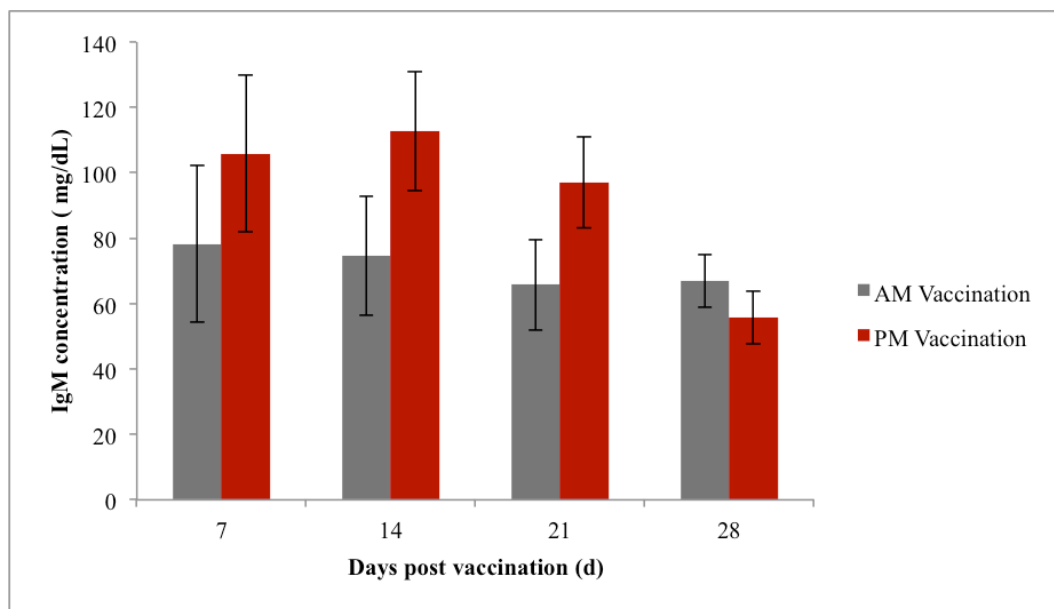


Figure 5: Serum IgM concentrations in mg/dL from D7 through D28 post vaccination (n=4 per time point per group). Bars represent least square mean \pm SE. Day P=0.0157; Vaccination Time (VxTime) P=0.3378; Day*VxTime P=0.0294.

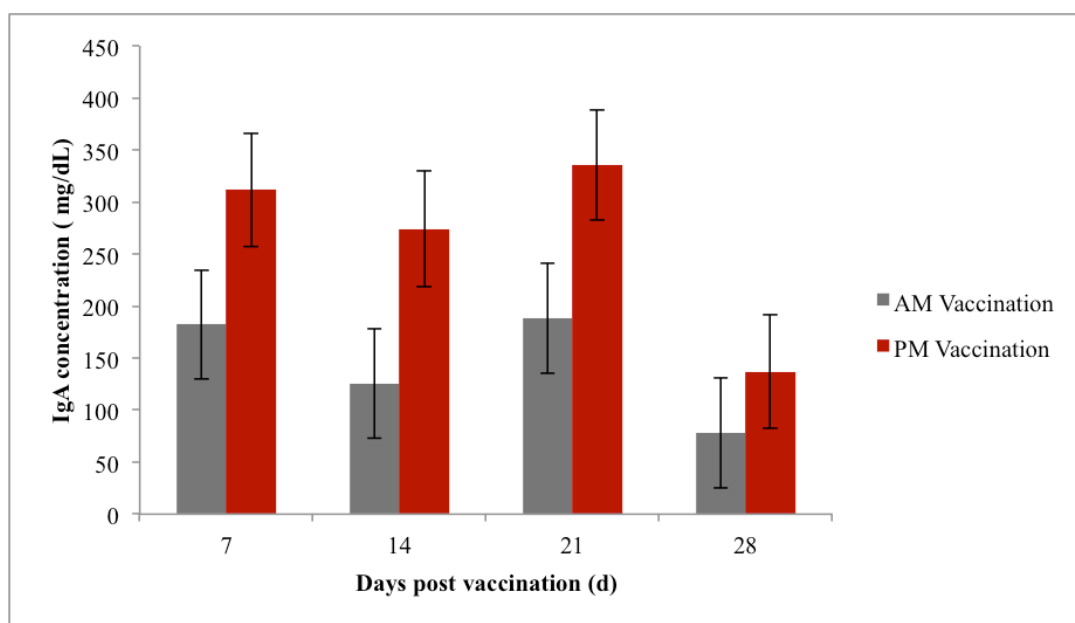


Figure 6: Serum IgA concentrations in mg/dL from D7 through D28 post vaccination (n=4 per time point per group). Bars represent least square mean \pm SE. Day P=0.0021; Vaccination Time (VxTime) P=0.1060; Day*VxTime P=0.5787.